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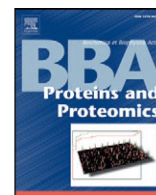

Review

The role of dynamics in modulating ligand exchange in intracellular lipid binding proteins

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ABSTRACT

Lipids are essential for many biological processes and crucial in the pathogenesis of several diseases. Intracellular lipid-binding proteins (iLBPs) provide mobile hydrophobic binding sites that allow hydrophobic or amphipathic lipid molecules to penetrate into and across aqueous layers. Thus iLBPs mediate the lipid transport within the cell and participate to a spectrum of tissue-specific pathways involved in lipid homeostasis. Structural studies have shown that iLBPs' binding sites are inaccessible from the bulk, implying that substrate binding should involve a conformational change able to produce a ligand entry portal. Many studies have been reported in the last two decades on iLBPs indicating that their dynamics play a pivotal role in regulating ligand binding and targeted release. The ensemble of reported data has not been reviewed until today. This review is thus intended to summarize and possibly generalize the results up to now described, providing a picture which could help to identify the missing notions necessary to improve our understanding of the role of dynamics in iLBPs' molecular recognition. Such notions would clarify the chemistry of lipid binding to iLBPs and set the basis for the development of new drugs.

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1. Introduction to the iLBP protein family and role of dynamics in their function

Lipidic molecules such as fatty acids, eicosanoids and bile salts are essential for cell survival because they serve as metabolic energy sources, substrates for membranes and signaling molecules for metabolic regulation [1–3]. The poor solubility of most of these lipids and in some cases their cytotoxicity require that intracellular chaperones bind them with high affinity and transfer them through the aqueous cellular environment. To better understand the action of lipids it is essential to gain a detailed knowledge on the mechanisms of their interactions with intracellular cognate binding proteins [4].

Lipidic compounds are bound reversibly by small soluble protein carriers, called intracellular lipid binding proteins (iLBPs), that target their ligands specifically to cellular compartments, including the peroxisomes, mitochondria, endoplasmic reticulum and nucleus [5,6]. iLBPs are a large protein family, which includes fatty acid (FABP), cellular retinol (CRBP), cellular retinoic acid (CRABP) and bile acid (BABP) binding proteins. These proteins are widely distributed throughout the body; they have distinct tissue distributions and exhibit a different degree of binding promiscuity. Due to their central role in lipid-mediated biological processes and systemic metabolic homeostasis [7,8], iLBPs have been proposed as therapeutic targets against lipid-related disorders.

Phylogenetic analysis divided the iLBP family into four subfamilies: subfamily I including CRBPs and CRABPs, subfamily II including BABPs and liver-FABP, subfamily III including intestinal-FABP and subfamily IV including all the remaining FABPs [9] (Fig. 1).

Despite differentiation based on primary structure, all iLBPs share the same overall topology consisting of a β-barrel, formed by ten anti-parallel β strands (A–J), containing a ligand binding N-terminal helix(αI)–turn–helix(αII) motif. Members of subfamilies I, III and IV were reported to bind only one ligand per molecule of protein, while proteins belonging to subfamily II can bind two ligands simultaneously.

Structural studies have shown that iLBPs have binding sites inaccessible from the bulk, implying that substrate binding should involve a conformational change able to produce a ligand entry portal. However, by comparison of the structures determined for unbound and ligand-bound proteins, it was found that ligand-loaded iLBPs have very similar structures to those of unbound states. This notion leads to the suggestion that protein internal dynamics influences the mechanism of ligand entry and exit as well as ligand binding preferences. While the mechanisms of ligand exchange remain largely unknown, the ligand binding process was described for some FABPs in terms of a "dynamic portal hypothesis", whereby the mobility of the helix cap would modulate ligand entry and release [10–12]. We anticipate that this early hypothesis

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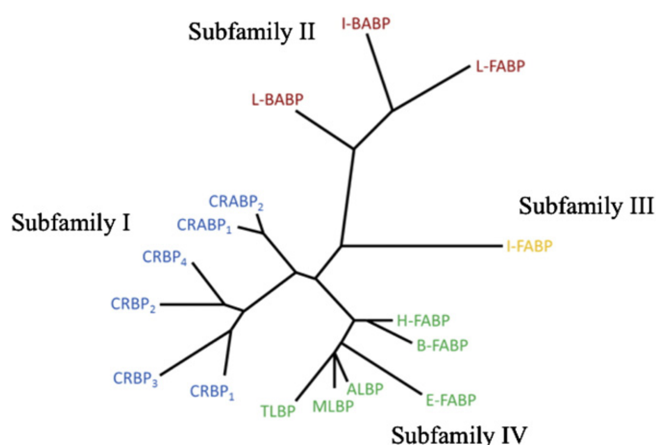

Fig. 1. Phylogenetic analysis of intracellular lipid binding protein family. The classification of the different subfamilies is reported. CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; I-BABP, intestinal bile acid binding protein; L-BABP, liver bile acid binding protein; L-FABP, liver fatty acid binding protein; I-FABP, intestinal fatty acid binding protein; H-FABP, heart fatty acid binding protein, B-FABP, brain fatty acid binding protein; E-FABP, epidermal fatty acid binding protein, ALBP, adipocyte fatty acid binding protein, MLBP, muscle fatty acid binding protein; TLBP, testis fatty acid binding protein.

was later found not sufficient to explain all aspects of ligand binding by iLBPs, however a significant number of independent studies can be reconciled considering that substrate binding by iLBPs is intrinsically linked to protein dynamics.

The ensemble of dynamics studies performed on different iLBPs in the last two decades is here reviewed to address the general mechanism of protein action. Besides providing an overview that may contribute to shed light into iLBP function and lipid trafficking, the subject also bears relevance for the development of pharmaceutical agents that could modify FABPs' function to control lipid signaling pathways, inflammatory responses and metabolic regulation. NMR spectroscopy has been the prominent technique to investigate protein dynamics for its capability to deliver information at atomic resolution over a wide range of timescales, ranging from picoseconds to seconds that characterize protein internal motions with functional relevance. These include conformational fluctuations occurring during enzymatic activities, protein folding and regulation, and signaling [13]. As a theoretical framework is needed to extract dynamics information from NMR observables, the first section of this review will provide a brief outline of NMR methods in protein dynamics to introduce technical terminology used in the subsequent discussion [14]. The iLBPs' dynamics data, here reported, are summarized based on the motion timescales they refer to and on their correlation with functional aspects.

2. General aspects of NMR approaches for deriving different timescale protein dynamics

Fast timescale dynamics define fluctuations among a large ensemble of structurally similar states that are separated by energy barriers of less than 1 kT (the product of the Boltzmann constant and the absolute temperature). These are small-amplitude sub-nanosecond motions experienced by disordered loops or describing side chain rotations. The fast sampling of substates has important implications for the entropy of the target protein, which involves a very large number of degrees of freedom and whose estimate certainly represents one of the greatest challenges [13,15]. Fast fluctuations are universal and have also been proposed to facilitate exchange events on slower timescales [16,17]. Changes in small-amplitude atomic motions upon ligand binding can also influence the free energy of molecular associations [17,18].

Rapid, random fluctuations impose time modulation on local magnetic fields that cause nuclear spins to relax to equilibrium after they are excited in an NMR experiment. A careful measurement of NMR relaxation times can thus reveal the timescale and amplitude of fast local motions. Three relaxation parameters for the ^{15}N–^{1}H pair, longitudinal relaxation R_1, transverse relaxation, R_2, and ^{1}H–^{15}N NOE [19] are usually measured. R_1 and ^{1}H–^{15}N NOE report directly on motions on the fast timescale, while R_2 additionally reports on slower motions. The relaxation of magnetization to equilibrium, after excitation, is governed by global macromolecular tumbling as well as internal motion. A mechanistic understanding of protein dynamics requires separation of these contributions. In this regard, a popular approach relies on the so-called "model-free" formalism [20], which assumes that the two types of motion are independent of each other and separated in timescale. In case of spherically shaped molecules, overall motion is described as isotropic and defined by a single correlation time, τ_c. Additional complexity in terms of axially symmetric or fully anisotropic rotational diffusion models may be required, depending on the protein's hydrodynamics. The basic site-specific parameters that are adjusted to fit the relaxation data are the squared generalized order parameter, S^2, which measures the amplitude of local motions, and the effective correlation time, τ_e, which defines the timescale of bond vector reorientation. S^2 are often mapped to the protein sequence to identify local flexibility. They have limiting values of unity, corresponding to complete rigidity, and zero, corresponding to unconstrained isotropic motion. NMR dynamics data on fast timescales can generally be complemented by *in silico* protein dynamics simulations.

Slow timescale fluctuations occur between kinetically distinct states that are separated by energy barriers of several kT units, corresponding to timescales of microseconds or slower. Ligand binding and release, protein folding/unfolding, allosteric processes, and catalytic turnover of enzymes may be affected, among others, by amino acid side chain reorientations, loop motions, and secondary structure changes, indeed occurring on micro-millisecond timescales. A dynamic process that exposes an NMR probe to at least two distinct chemical environments, or states, in a time-dependent manner is referred to as chemical exchange [13]. Chemical exchange is classified into three distinct regimes, defined by the comparison of the exchange rate k_{ex} and $|\Delta\nu|$, the difference in resonance frequency of a nucleus between two conformational states. The three regimes are denoted as slow ($k_{ex} \ll |\Delta\nu|$), intermediate ($k_{ex} \approx |\Delta\nu|$) and fast ($k_{ex} \gg |\Delta\nu|$). Exchange phenomena occurring at a rate k_{ex} similar to $\Delta\nu$ result in an increased relaxation rate and an exchange-broadened signal with $R_2^{obs} = R_2° + R_{ex}$ (where $R_2°$ is the transverse relaxation rate constant in the absence of exchange and R_{ex} accounts for the exchange contribution). The presence of slow motions necessitates the fitting of ^{15}N NMR relaxation data with an extended model-free formalism that includes the additional adjustable parameter R_{ex} [21]. However, unlike S^2, R_{ex} terms are often interpreted semi-quantitatively and mostly help to direct more detailed studies [22,23]. Quantitative analysis of slow dynamics in proteins is based on more sophisticated NMR relaxation dispersion (RD) experiments, which include the Carr–Purcell Meiboom–Gill (CPMG) and rotating frame (RF) approaches [24]. RF-RD can be used to study exchange events in a faster time window (~20–100 μs) than CPMG-RD (~0.3–10 ms) but is less powerful to derive kinetic and thermodynamic information and is more demanding for NMR hardware. The principle of CPMG-RD is to refocus exchange broadening or to reduce R_{ex} in a controlled fashion by applying spin-echo pulse elements with different inter-pulse delays (CPMG frequency, ν_{CP}). The dependence of R_2^{obs} on ν_{CP} can be fit to extract exchange parameters, depending on the exchange regime. CPMG-RD experiments have become popular due to the fact that they allow obtaining information even on very low populated excited conformational states, sometimes revealing invisible functional states [25]. The RD methods, together with lineshape analysis, allow probing the kinetic, thermodynamic and structural parameters that define conformational fluctuations [26].

Slower motions, occurring with time constants of milliseconds to seconds, can be monitored using hydrogen–deuterium exchange

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